DISTRIBUTION OF APGWamide-LIKE AND FMRFamide-LIKE IMMUNOREACTIVE NEURONS INNERVATING THE PENIS AND THE DART SAC IN THE MESOCEREBRUM OF THE SNAIL *HELIX ASPERSA*

by

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Axon projections and peptide content in mesocerebrum of Helix aspersa.

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ABSTRACT

The distribution of APGWamide-like and FMRFamide-like immunoreactive neurons involved in the mating behaviour of the terrestrial snail *Helix aspersa* have been investigated in the mesocerebrum by retrograde and anterograde labelling and immunostaining techniques. Retrograde labelling shows that 25-40 mesocerebral neurons have an axon in the penis nerve and a similar number of neurons have an axon in the nervus cutaneus pedalis primus dexter (NCPD). It was found that the mesocerebral neurons also project to the nervus cutaneus pedalis secundus dexter (NCSD), the medial lip nerves, the posterior lip nerve, the peritentacular nerve, and the pedal ganglion. Some mesocerebral neurons have multiple projections. Quantitative analysis using confocal laser scanning microscopy indicates that approximately half of the mesocerebral neurons projecting to the penis nerve contain APGWamide-like peptide and half of the mesocerebral neurons projecting to the NCPD contain FMRFamide-like peptide. Some mesocerebral neurons have both peptides. These results are generally in agreement with the hypothesis that the neurons projecting to the penis nerve contain APGWamide, while those projecting to the NCPD contain FMRFamide.

RÉSUMÉ

A l'aide de techniques de marquage par rétrogradation et antérogradation, la distribution des neurones immunoréactives aux anticorps anti-APGWamide et anti-FMRFamide, impliquées dans le comportement d'accouplement chez l'escargot *Helix aspersa*, a été étudiée. Le marquage par rétrogradation démontre que de 25 à 40 neurones du mésocérébrum ont leur axone dans le nerf du pénis, tandis qu'environ le même nombre ont leur axone dans le nervus cutaneus pedalis primus dexter (NCPD). Il a été observé que des neurones du mésocérébrum ont aussi des prolongements dans le nervus cutaneus pedalis secundus dexter (NCSD), les nerfs médiaux et postérieur des lèvres, le nerf péritentaculaire, et le ganglion pédale. Quelques unes des neurones du mésocérébrum ont plusieurs prolongements. A l'aide du "confocal laser scanning microscope", une analyse quantitative des neurones du mésocérébrum a été effectuée: la moitié des cellules ayant leur axone dans le nerf du pénis sont immunoréactives aux anticorps anti-APGWamide, de même que la moitié de celle ayant leur axone dans le NCPD sont immunoréactives aux anticops anti-FMRFamide. De plus, quelques neurones du mésocérébrum sont immunoréactives aux deux anticorps. Ces résultats sont en accord avec l'hypothèse que les neurones ayant un prolongement dans le nerf du pénis et dans le NCPD contiennent les peptides APGWamide et FMRFamide, respectivement.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

To the best of my knowledge, the experimental results presented in this thesis on distribution in the mesocerebrum of APGWamide-like and FMRFamide-like immunoreactive neurons innervating the penis and the dart sac are contributions to original knowledge. This study provides a better understanding of morphology of the neurons in the right mesocerebrum that play an important role in controlling penis eversion and dart shooting during courtship and mating. The peptide contents of the right mesocerebral neurons have been determined, and the relationship between axon projections of the right mesocerebral neurons and the peptides they contain has been established.

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Figure 6, 7, 8, 9, 10, 12, 13, 15 are photocopies of original color photographs.

Chapter 1

Introduction

The gross morphology of the brain of terrestrial pulmonate gastropods has been fully examined by many earlier investigators (see Chase, 1986). However, neuroethology of terrestrial snail has only been studied to a limited extent, in spite of their relatively simple nervous system and their possession of many large and accessible neurons. In recent years, the mating behaviour and its neural control of the terrestrial snails *Helix* have been published (Adamo and Chase, 1988, Chase, 1986, Eberhardt and Wabnitz, 1979). Chase (1986) reported that in *Helix aspersa* the mesocerebrum of the brain was involved in controlling mating behaviour. More recently, there are reports that the mesocerebral neurons contain neuropeptides Ala-Pro-Gly-Trp-NH_2 (APGWamide) (Griffond et al., 1992) and Phe-Met-Arg-Phe-NH_2 (FMRFamide) (Marchand et al., 1991, Elekes and Nässel, 1990). The purpose of the present study is to investigate the topographical distribution of APGWamide-like and FMRFamide-like immunoreactive neurons involved in mating behaviour in the mesocerebrum of *Helix aspersa*.

The garden snail *Helix aspersa* is a simultaneously reciprocal hermaphrodite. Each adult possesses all the nervous and reproductive systems necessary for mating activity in both male and female roles. The copulatory organs are situated on the right side of the animal. Mating behavior in *Helix aspersa* is a complex process, which includes three major phases: introductory behaviour, dart shooting, and copulation (Adamo and Chase, 1988).

Introductory behaviour includes reciprocal tactile and oral contacts. After oral contact, the genital apparatus begins to evert, i.e., an increasing amount of normally internal genital atrium is extended outside the body wall. The eversion has six stages and can differ from stage 0 to 5 during introductory behaviour. Dart shooting occur when a sharp, hollow calcareous dart (Hunt, 1979) pierces the body of the mating partner. The calcareous dart is secreted in the dart sac, a structure with thick muscular walls. It is attached by its base to the papilla in the base of the dart sac (Adamo and Chase, 1988, Chung, 1987). Dart shooting is accomplished by internal hydrostatic pressure and muscular contractions, and the dart is expelled by eversion of the papilla. The everting tissue pushes the dart out of its sac into the flesh of the partner. Once lodged in the partner, the dart is detached from the papilla and is left in the partner (Chung, 1987). The dart fully regenerates within 5 to 7 days after dart shooting (Tompa, 1984). It is believed that dart shooting may facilitate mating by increasing behavioural synchrony (Adamo and Chase, 1988).

Copulation occurs immediately or shortly after dart shooting, i.e., the penis is fully everted at the genital pore and there are intromission and reciprocal sperm transfer. After eversion, the extruded penis is withdrawn back into the body by a specialized muscle, the penis retractor muscle (Tompa, 1984, Eberhardt and Wabnitz, 1979). Although the mechanism of penis eversion has not been fully

understood, it is believed that in *Aplysia* the penis retractor muscle retains the penis and its sheath within the body until copulation occurs, whereupon the penis and the sheath are everted from the animal and the penis retractor muscle is extended three or more times of its normal resting length (Rock et al., 1977).

The central nervous system of *Helix aspersa* consists of a circumesophageal ring of two supraesophageal (cerebral) and seven subesophageal ganglia (Kerkut et al., 1975). The cerebral ganglia are bilaterally symmetric and made up of three parts: the procerebrum, the mesocerebrum, and the postcerebrum (Figure 1). The cerebral ganglia are connected by the cerebral commissure. The mesocerebrum exhibits a right-sided bias in that the right lobe has 23% more neurons than the left lobe and the average cell size is 24% larger on the right lobe than on the left lobe (Chase, 1986).

It is known that the penis is innervated by the penis nerve (PN), which emerges from the right cerebral ganglion near the right medial lip nerve (Goddard, 1962), while the dart sac is innervated mainly by the nervus cutaneus pedalis primus dexter (NCPD)(Schmalz, 1914), which emerges from the right pedal ganglion very close to the right cerebropedal connective (CPC), but sometimes the two nerves are fused at the point of attachment.

Morphological and physiological evidence indicates that the mesocerebral neurons are involved in the control of sexual behaviour (Chase, 1986). Extracellular stimulation

Figure 1. Dorsal view of the central nervous system of *Helix aspersa*.

Note the nerves (PN and NCPD) that innervate the male reproductive organs (the penis and the dart sac).

CC, cerebral commissure.

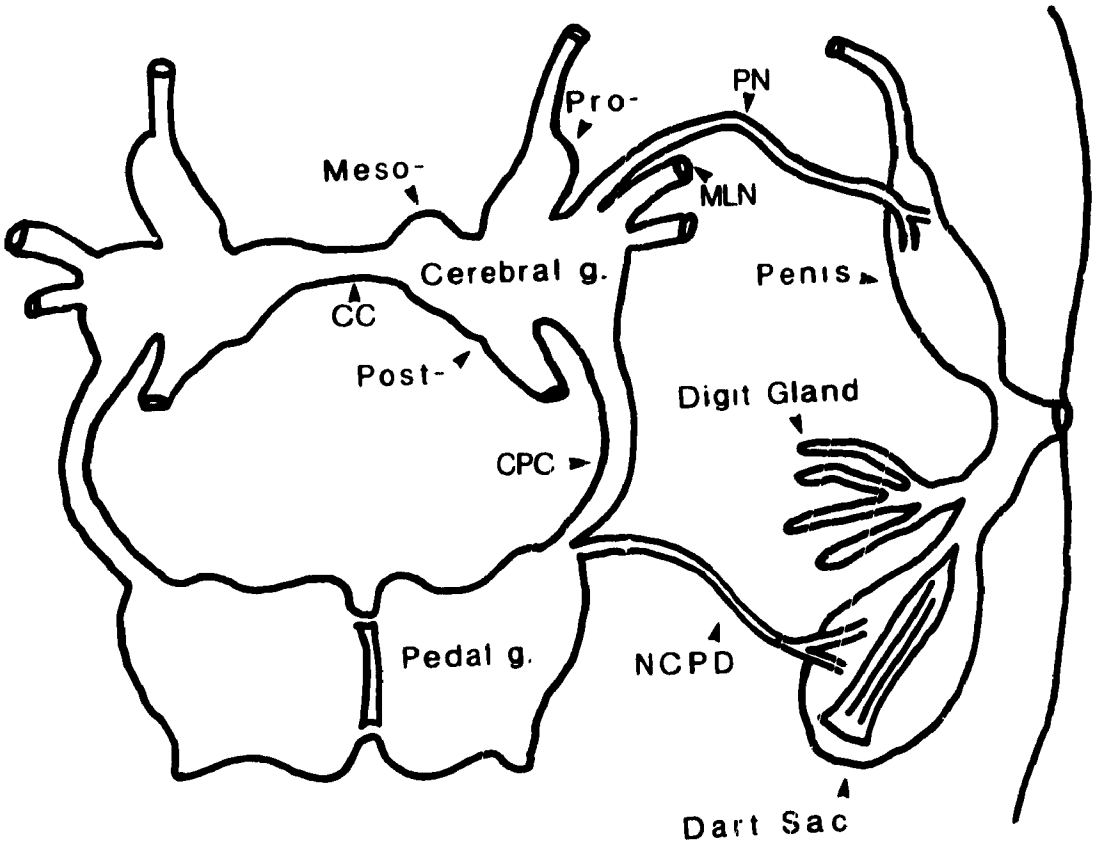
CPC, cerebropedal connective.

MLN, medial lip nerve.

NCPD, nervus cutaneus pedalis primus dexter.

PN, penis nerve.

(Drawn by R. Chase and presented in the Third International Congress of Neuroethology, Montreal, 1992).


Pro-
PN
Meso-
MLN
Cerebral g.
Penis
CC
Post-
Digit Gland
CPC
Pedal g.
NCPD
Dart Sac

of the right mesocerebrum resulted in the movements of the penis and the dart sac, while intracellular stimulation of some mesocerebral neurons produced large contractions of the dart sac and the penis sheath. These movements are similar to those required, in an intact snail, to release the dart and evert the penis. This evidence indicates that the right mesocerebral neurons are capable of commanding penis eversion and dart release (Chase, 1986).

It has been identified, from immunohistochemistry, that in *Helix*, two major peptides in the mesocerebrum are APGWamide (Griffond et al., 1992) and FMRFamide (Marchand, et al., 1991, Elekes and Nässel, 1990). In *Helix aspersa*, APGWamide immunoreactive neurons were observed in the mesocerebrum and immunoreactive fibres were detected in the penis but not in the dart sac (Griffond et al., 1992). Griffond and co-workers reported that APGWamide positive neurons were located in the anterior lobe of the right mesocerebrum but not in the left mesocerebrum. The axons of these APGWamide neurons formed a bundle running across the right cerebral ganglion to the origin of the right cerebropleural connective (CPLC) and the right cerebropedal connective. APGWamide immunoreactive fibres were found in some nerve roots such as the penis nerve, and in the muscular layer of the penis, especially in the proximal half. They suggested that the APGWamide is probably involved in the contraction or the relaxation of the muscular layer of penis during copulation.

The tetrapeptide APGWamide was first found and purified from the prosobranch mollusc *Fusinus ferrugineus* (Kuroki et al., 1990). The primary structure of this peptide is closely related to the C-terminal tetrapeptide fragment of the crustacean red pigment concentrating hormone, RPCH. APGWamide has stimulatory or inhibitory effects on various mollusc muscles (Muneoka et al., 1991, Kuroki et al., 1990, Kobayashi and Muneoka, 1990). APGWamide potentiated twitch contractions of the radula retactor of *Fusinus* in response to short electrical pulses of stimulation, whereas the peptide inhibited twitch contractions of the radula protractor. APGWamide also potentiated twitch contractions of the radula retractor of *Rapana*, but it inhibited contraction in a number of muscles, such as, the anterior byssus retractor muscle and the pedal retractor of *Mytilus*, the crop and the pharyngeal retractor of *Euhadra*.

In *Lymnaea stagnalis*, it is known that the neurons innervating the penis are located mostly in the anterior lobe and the ventral lobe of the right cerebral ganglia, and the right pedal ganglion (Croll et al., 1991, Van Duivenboden, 1984). APGWamide immunoreactive neurons were detected mainly in the right anterior lobe, whereas fewer neurons were located in the smaller left anterior lobe. Consistently strong hybridization signals with the oligonucleotide probe for APGWamide encoding mRNA were observed in the neurons of both the right and the left anterior lobes (Croll and Van Minnen, 1992). In addition,

Smit et al. (1992) have recently isolated and characterized a cDNA clone that encoded multiple copies of APGWamide expressed in the anterior lobe of the right cerebral ganglion. APGWamide positive fibres were found to locate in the penis retractor muscle, along the inner surface of the penis sheath and throughout the preputium (Croll and Van Minnen, 1992, Croll et al., 1991). Croll and co-worker suggested that APGWamide may play an important role in controlling the penis complex such as relaxation of the penis retractor muscle during the penis eversion.

The mesocerebrum of *Helix* is in many respects similar to the anterior lobe of *Lymnaea*. Both the right mesocerebrum and the right anterior lobe have more neurons and are larger than their counterparts in the left side. Similar to the right anterior lobe of *Lymnaea*, the neurons of the right mesocerebrum of *Helix* innervate the penis (Chase, 1986). However, the latter innervate the penis indirectly through pedal neurons. Since APGWamide immunoreactivity was found in both right anterior lobe in *Lymnaea* (Croll and Van Minnen, 1992) and the right mesocerebrum in *Helix* (Griffond et al., 1992), it seems reasonable to assume that in *Helix* the mesocerebral neurons projecting to the penis contain APGWamide, which has similar function as in *Lymnaea*.

FMRFamide was first isolated and sequenced from the clam *Macrocallista nimbosa* (Price and Greenberg, 1977A, B), FMRFamide and related peptides have since been found not

only in a number of gastropod species, such as *Lymnaea* (Benjamin et al., 1988, Schot and Boer, 1982), *Aplysia* (Lloyd et al., 1987), *Achatina* (Takayanagi and Takeda, 1987), *Helisoma* (Bulloch et al., 1988, Saleuddin et al., 1992), *Limax* (Cooke and Gelperin, 1988), and *Helix* (Elekes and Nässel, 1990), but also throughout the animal kingdom, including insect and fish (Boer et al., 1980), hydra (Grimmelikhuijzen et at., 1982), and vertebrates (Dockray et al., 1981). There is evidence that FMRFamide has potent effects on cardiac muscle, smooth muscle, and neurons in mollusc (Lehman and Greenberg, 1987, Cottrell, 1982). Not only FMRFamide but six other related peptides have also been found in *Helix* (Price, 1990, Cottrell, 1989, Price et al., 1985). They are: FMRFamide, FLRFamide, pQDPFLRFamide, NDPFLRFamide, SDPFLRFamide, SEPYLRFamide, NDPYLRFamide. FMRFamide and FLRFamide have virtually identical activities on various mollusc tissues, while the heptapeptides have biological effects different from the tetrapeptides on some pulmonate muscles (Price, 1986). In *Helix*, FMRFamide causes the tentacle retractor muscle to contract, while pQDPFLRFamide causes the muscle to relax. However, there is evidence that both FMRFamide and pQDPFLRFamide contracted the male reproductive tract, including the dart sac, which implies that these peptides could control the reproductive behaviour (Lehman and Greenberg, 1987).

Elekes and Nässel (1990) detected that in *Helix pomatia* medium-sized FMRFamide immunoreactive neurons were situated

mostly in the outer layer of the mesocerebrum. Varicose FMRFamide positive fibres were visualized in the cell body layer of the different ganglia, and in the neuronal sheath of both the ganglia and the peripheral nerves. Similarly, in *Helix aspersa*, there was evidence of existence of FMRFamide immunoreactivity in the mesocerebrum (Marchand et al., 1991). It was also reported that the thick muscular wall of the dart sac was densely innervated by FMRFamide immunoreactive fibres (Cardot, 1983).

This review of the literature shows that the mesocerebral neurons are capable of commanding penis eversion and dart release, and the mesocerebrum contains APGWamide and FMRFamide immunoreactive substances. Additionally, APGWamide immunoreactive fibres are present in the penis sheath and the penis retractor muscle but not in the dart sac. FMRFamide immunoreactive fibres are strongly present in the thick muscular wall of the dart sac but only lightly present in the penis retractor muscle (Lehman and Price, 1987, Cardot, 1983).

To elucidate the relationship between the mesocerebral neurons innervating the penis or the dart sac and the peptide they contain, a hypothesis was raised based on the aforementioned immunohistochemical and electrophysiological evidence: the mesocerebral neurons projecting to the penis nerve contain APGWamide, while the mesocerebral neurons projecting to the NCPD contain FMRFamide. A double label study was conducted to test this hypothesis. The penis

nerve or the NCPD was backfilled with Neurobiotin, and then conjugated with rhodamine-avidin so that the mesocerebral neurons projecting to either nerve would be labelled with rhodamine and yield red color. Tissue sections were subsequently immunoreacted with antisera for APGWamide or FMRFamide, later reacted to fluorescein isothiocyanate (FITC) conjugated secondary antibodies. The immunoreactive neurons would yield yellow/green color, while the backfilled and immunoreactive neurons (double label) would result in orange color. From the color of the labelled mesocerebral cells, one can examine the relationship between the projections of the mesocerebral neurons and their peptide content.

Based on the preliminary results of retrograde labelling, an anterograde labelling was subsequently conducted to correlate the projections of individual neurons in the right mesocerebrum with the peptide they contain. To detect their peptide content, alternative sections of labelled neurons were immunoreacted with antisera for either APGWamide or FMRFamide. Anterograde labelling can not only allow one to examine the cases where neurons have multiple projections, but also to confirm the backfill results since the axon pathway can be unambiguously identified.

Chapter 2

Materials and Methods

Animals

Adult snails *Helix aspersa* in the range of 5-9 g were either obtained from California or cultured in this laboratory. They were maintained on a light/dark cycle of 14/10 hours and fed lettuce and rat chow. The circumesophageal ganglia were removed from the snails and placed in a snail saline consisting of 80 mM NaCl, 8 mM $CaCl_2$, 5 mM $MgCl_2$, 4 mM KCl, and 5 mM Tris buffer at pH 7.8. The ganglia were pinned out in a Sylgard dish. The penis nerve and the NCPD were dissected free from the surrounding connective tissue. For intracellular injections, the right mesocerebrum was also desheathed. All animals were sacrificed during behaviourally active stage. For reconstruction using confocal laser scanning microscopy, three snails were sacrificed during the genital eversion stage 3 and one snails was sacrificed at the genital eversion stage 1 according to the criteria used by Adamo and Chase (1988).

Retrograde Labelling

Three compounds were used as retrograde labelling agents: hexaminecobalt chloride, horseradish peroxidase (HRP), and Neurobiotin (N-(2-aminoethyl) biotinamide hydrochloride)(Vector Lab.). The cut end of the penis nerve or the NCPD, about 0.5 mm in length, was sucked into a fitted glass pipette containing 0.2 M hexaminecobalt chloride and left there for 6 to 10 hours. After the nerve was removed from the pipette, the preparation was processed

with H_2S and intensified with silver following the method of Davis (Chase and Tolloczko, 1993, Davis, 1982).

The penis nerve backfilled with 15% horseradish peroxidase was performed in a similar fashion as with hexaminecobalt chloride. Development with 0.5% benzidine dihydrochloride (BDHC) and subsequent processings of the HRP reaction were carried out using the method of Muller and McMahan (1976).

The labelling with Neurobiotin was processed as follow: the penis nerve (total n=19) or the NCPD (total n=12) was backfilled with 8% Neurobiotin in 0.1 M Tris buffer (pH 7.6). The backfill times were 8-9 hours for the penis nerve, and 16-17 hours for the NCPD. The preparations were then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 8-16 hours at 4°c. After washing with PB, the preparations were processed with the avidin-biotin peroxidase method using the Vectastain ABC Kit (Vector Lab.). The preparation was immersed in Vectastain ABC plus 0.3% Triton X-100 in PB for 4 hours. After washing with fresh PB, the preparation was reacted with 0.05% diaminobenzidine tetrahydrochloride (DAB) plus 2% cobalt chloride. Finally, the preparation was dehydrated in a series of 25%, 50%, 75%, 90%, 100% ethanol and cleared in methyl salicylate. The specimens were viewed and photographed under a Zeiss light microscope.

Anterograde Labelling

For anterograde labelling, large (>50 µm) individual

cells (one cell per brain) of the right mesocerebrum were injected with 8% Neurobiotin in 1 M potassium chloride by passing 0.5-1.0 nA depolarizing current for 10-60 minutes using 500 ms pulses at 1 Hz. Microelectrodes of 30-100 MΩ were chosen for injection. This procedure was modified from that provided by Vector Lab. After the injection, the circumesophageal ganglia were cut into two parts, between the right anterior lip nerve and the penis nerve.

The first part of circumesophageal ganglia which contained the penis nerve, the right postcerebrum, the right CPC, the right CPLC, the entire subesophageal ganglia and the NCPD was subsequently processed with the ABC procedure as described previously. The specimen was then viewed as a whole mount under a Zeiss light microscope and the axon of the injected cell was traced using a drawing tube attached to the microscope.

The remaining piece of the cerebral ganglion, which contained the procerebrum, the mesocerebrum and the left postcerebrum but neither the penis nerve nor the right CPC, was subsequently incubated in rhodamine$_{600}$-avidin D (Vector Lab.) diluted 1:100 in carbonate buffer (pH 8.6) for 4-24 hours and infiltrated with 30% sucrose in 0.1 M PB. The preparation was frozen and then sectioned at 10-15 µm in a cryostat. Alternative sections were immunoreacted to either anti-APGWamide or anti-FMRFamide antiserum and further processed as described below. The immunohistochemical procedure was modified from the methods of Cooke and

Gelperin (1988) and of Croll and Van Minnen (1992).

Immunohistochemistry

A double labelling was carried out in single brains. The projection of mesocerebral neurons was revealed by backfilling the penis nerve or the NCPD with Neurobiotin, visualized by subsequently reacting to rhodamine-avidin which yielded a red colour. The peptide content in the backfilled mesocerebral neurons was determined by immunoreacting to either anti-APGWamide or anti-FMRFamide antiserum, and then reacting to FITC conjugated secondary antibodies which were green in colour.

After either the penis nerve or the NCPD was backfilled with Neurobiotin, the preparation was fixed and serially sectioned at 20-25 µm. The frozen sections were mounted on 3-aminopropyltriethoxy-silane (TES) coated slides and treated with 0.3% Triton X-100 in PB for 30 minutes. The sections were incubated in rhodamine-avidin diluted 1:200 in carbonate buffer for 2 hours. The incubation and all subsequent processes were performed at room temperature.

For the immunostaining of anti-FMRFamide, the sections were incubated in 10% normal goat serum in 0.1 M PB for 1 hour. They were incubated in rabbit anti-FMRFamide antiserum (Peninsula Lab.) diluted 1:500 in PB plus 1% normal goat serum for 2 hours. The sections were then incubated in FITC conjugated goat anti-rabbit IgG (Sigma) diluted 1:20 in PB for 1 hour.

For the immunostaining of anti-APGWamide, the sections

were incubated in 10% normal rabbit serum and reacted to guinea pig anti-APGWamide antiserum (kindly provided by Dr. Jan Van Minnen) diluted 1:500 in PB plus 1% normal rabbit serum. Following this the sections were incubated in FITC conjugated rabbit anti-guinea pig IgG (Zymed Lab.) diluted 1:20 in PB for 1 hour.

Some of the preparations reported in this study were immunoreacted as a whole mount. Before being fixed, the preparations were treated with 0.5% protease (Sigma) in 0.1 M PB for 5-7 minutes at room temperature. The preparations were then fixed in 4% paraformaldehyde in PB for 16-24 hours and immersed in 0.1 M PB plus 4% Triton X-100 and 1% normal rabbit serum (PTS) for 24 hours. Fixation and all subsequent steps were carried out at 4 °C. The ganglia were next incubated in anti-APGWamide antiserum diluted 1:1000 in PTS for 72 hours. After being washed in 0.1 M PB plus 4% Triton X-100 for 12 hours, the ganglia were further reacted to 1:50 diluted FITC conjugated rabbit anti-guinea pig IgG in PB with 2% Triton X-100 for 16 hours. Before viewing, the preparations were washed three times with fresh PB for 8-12 hours.

After immunoreaction, all sections or whole mounts were mounted in 90% glycerol in 0.1 M PB (pH 8.6) containing 0.1% phenylenediamine. The fluorescently labelled specimens were examined and photographed with either a Leitz epifluorescence microscope or a Leica confocal laser scanning microscope equipped with a 488 nm excitation filter

and a 510 nm barrier filter for FITC, or a 568 nm excitation filter and a 590 nm barrier filter for rhodamine. Quantitative analysis was carried out by scanning images of serial sections of a single brain, reconstructing the whole brain, and counting the immunoreactive cells.

The specificity of immunostaining was examined by preabsorbing the diluted antisera with either synthetic FMRFamide (Sigma) or APGWamide (Peninsula Lab.)(0.4 mg/ml of diluted antiserum) at 4°C for 24 hours before processing the tissue with antiserum.

Chapter 3

Results

Retrogradely labelled cells and fibres in the cerebral ganglia

Cells and fibres in the cerebral ganglia were labelled with Neurobiotin, hexaminecobalt chloride, and horseradish peroxidase. It was found that the three different methods yielded similar results. Retrograde labelling by the use of Neurobiotin from the penis nerve or the NCPD offers advantages over the other methods in that the labelled tissues can be further used in immunostaining and the background labelling is minimal. Typical examples of brains backfilled by Neurobiotin are given in Figure 2 and 3.

As illustrated in Figure 2, many cell bodies were backfilled from the penis nerve. They were mainly located in the following areas:

i) the anterior lobes of the mesocerebrum, mostly situated on the dorsal surface and at the medium level (between the dorsal and ventral surface), which included 25-40 cells (28-80 μm) in the right mesocerebrum and 10-20 cells (20-50 μm) in the left mesocerebrum,

ii) 25-45 cells (20-30 μm) in the right postcerebrum, and

iii) some cells (20-55 μm) scattered in the right CPC.

Additionally, a few cells were also found in the contralateral postcerebrum. However, no labelled cells were present in the procerebrum.

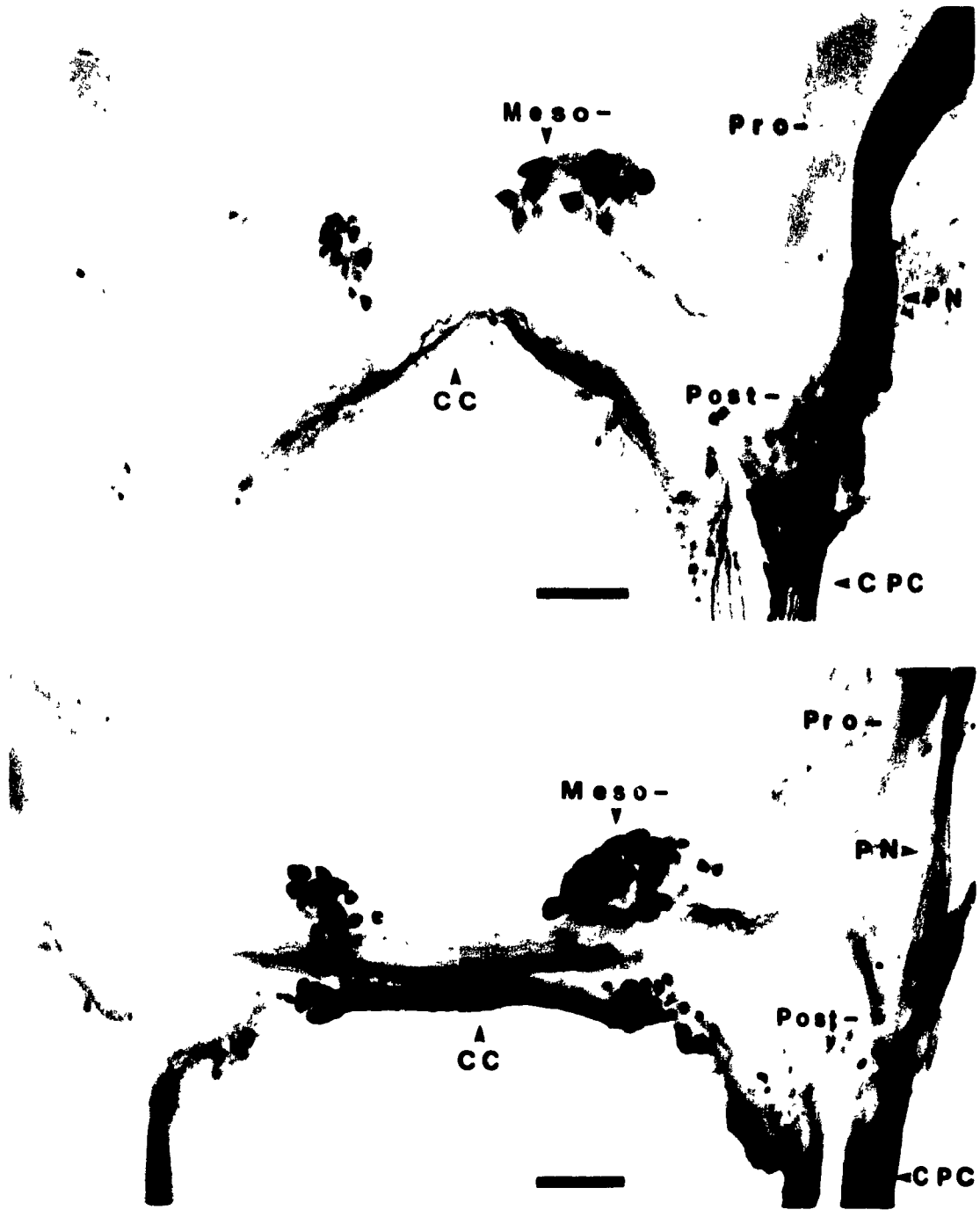
In addition to the labelled cells described above, retrogradely labelled axons were found extending to the ipsilateral pedal and pleural ganglia via the respective

Figure 2. Dorsal view of the cerebral ganglia backfilled from the penis nerve (PN).

Retrogradely labelled cells are mainly located in the mesocerebrum, the right postcerebrum, and the right cerebropedal connective (CPC). Note that fibres from the mesocerebrum and the CPC converge in the postcerebrum before exiting in the penis nerve. Also, note fibres in the cerebral commissure (CC). Scale bar = 200 µm.

Figure 3. Dorsal view of the cerebral ganglia backfilled from the nervus cutaneus pedalis primus dexter (NCPD).

Retrogradely labelled cells are primarily located in the mesocerebrum, the right postcerebrum, and the right cerebropedal connective. Note that fibres run into the right mesocerebrum. Also, note that fibres run across the cerebral commissure. Scale bar = 200 µm.


Meso-
Pro-
PN
CC
Post-
CPC
Pro-
Meso-
PN
Post-
CC
CPC

connective nerves. Another labelled axon pathway was seen to run into the left cerebropleural connective nerve via the cerebral commissure.

Backfills of the NCPD resulted in a pattern of labelled cells similar to that backfilled from the PN. In the mesocerebrum, labelled cell bodies were mostly located on the dorsal surface and at the medium level of the anterior lobes, including 25-50 cells (25-80 μm) in the right mesocerebrum and 12-34 cells (20-50 μm) in the left mesocerebrum (Figure 3). There were 20-45 labelled neurons (20-30 μm) in the right postcerebrum (Figure 4A) and a few neurons in the left postcerebrum. Another 40-80 labelled neurons (20-55 μm) were present in the right CPC, mostly located around the surface of the nerve (Figure 4B). A number of neurons (20-55 μm) were also located superficially on the right CPLC (Figure 4C). In addition, a total of over one hundred cells of different sizes were situated in the subesophageal ganglia, mostly located in the right pedal ganglion and the right pleural ganglion. The axon of these cells traveled throughout the entire ring of subesophageal ganglia. Another bundle of labelled fibres were found to ascend through the right connective nerves and cross the cerebral commissure and then enter the left CPC and the left CPLC.

Axon Projections of the right mesocerebral neurons

Anterogradely labelled cells (n=43) in the right mesocerebrum projected to the following areas: i) the right

Figure 4. Cell bodies and fibres in the right connective nerves filled by application of Neurobiotin to the NCPD.

A: Cell bodies in the right cerebropedal connective (CPC), and at the junction of the right CPC and the right postcerebrum (arrowhead).

B: Cell bodies and fibres in the right CPC. Cell bodies with long axons are located superficially on the nerve.

C: Cell bodies and fibres in the right cerebropleural connective (CPLC).

Scale bar = 50 µm.


A
B
C

CPC, ii) the penis nerve, iii) the NCPD via the right CPC, iv) the right medial lip nerve, v) the right posterior lip nerve, vi) the right peritentacular nerve, vii) the cerebral commissure, viii) the right pedal ganglion, and ix) the NCSD (nervus cutaneus pedalis secundus dexter) via the right CPC. Although most of the labelled cells projected to either the right CPC or the PN, some cells had multiple projections. An example is shown in Figure 5, in which the mesocerebral cell sends its axon into the right postcerebrum neuropil where it branches. One branch runs into the penis nerve. The second branch projects to the right CPC and the NCPD. The third branch runs into the right peritentacular nerve.

The quantitative results of axon projections of anterogradely labelled cells are given in Table 1. It should be noted that 98% of the labelled cells project to right CPC, 21% of the labelled cells project to the NCPD and 33% of the labelled cells project to the penis nerve. It should also be noted that 9% of labelled cells project to both the penis nerve and the NCPD. Since some cells have two or more branches projecting into different nerves, the total percentage of axons in these nerves is higher than 100%.

APGWamide-like immunoreactive cell bodies and fibres

The mesocerebral cells were retrogradely labelled from the penis nerve and visualized by reacting to rhodamine-avidin. The cerebral ganglia were then immunoreacted with APGWamide antiserum. Backfilled cells containing APGWamide-

Figure 5. Drawing of a mesocerebral cell projecting to the penis nerve, the NCPD, and the right peritentacular nerve (PTN). Scale bar = 200 µm.

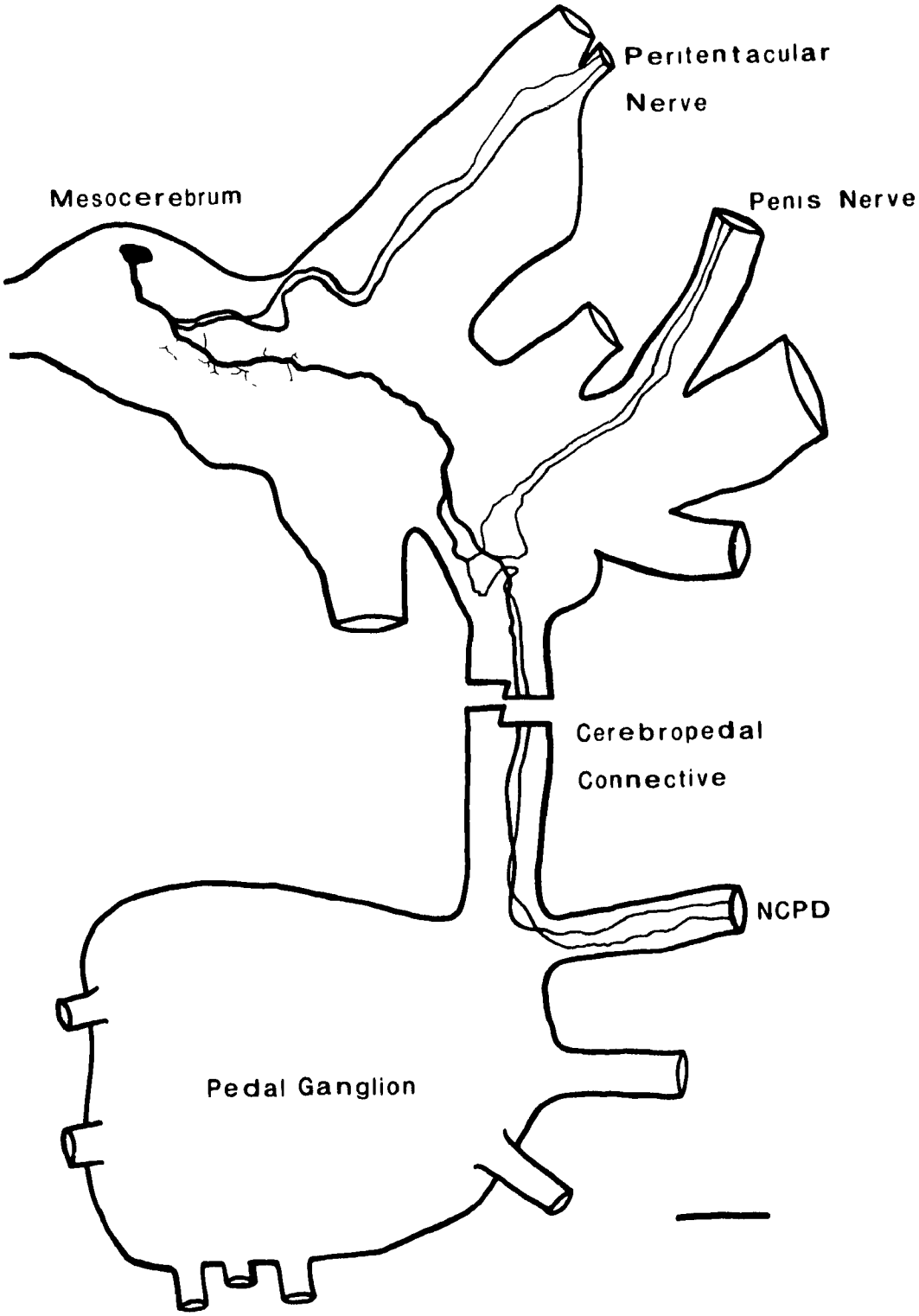

Peritentacular
Nerve
Mesocerebrum
Penis Nerve
Cerebropedal
Connective
NCPD
Pedal Ganglion

Table 1

Axon projections of the right mesocerebral neurons.

Nerves	Number of Neurons	Percentage (n=43)
CC	3	7%
CPC alone[a]	26	60%
CPC & NCPD	6	14%
CPC & NCSD	1	2%
CPC & Ped.g	6	14%
CPC & Ped.g & NCPD	3	7%
Total CPC	42	98%
Total NCPD	9	21%
MLN	6	14%
PLN	3	7%
PN	14	33%
PN & NCPD[b]	4	9%
PTN	3	7%

CC, cerebral commissure.
CPC, right cerebropedal connective.
MLN, right medial lip nerve.
NCPD, nervus cutaneus pedalis primus dexter.
NCSD, nervus cutaneus pedalis secundus dexter.
Ped.g, right pedal ganglion.
PLN, right posterior lip nerve.
PN, penis nerve.
PTN, right peritentacular nerve.

[a] Axon projecting to the CPC could not be traced to any further distance site.

[b] The number of mesocerebral neurons projecting to the PN and the NCPD has also been included under PN and Total NCPD.

like peptide were therefore double labelled and displayed orange color.

APGWamide-like immunoreactive cell bodies from the penis nerve were found not only in the right mesocerebrum but also in the left mesocerebrum (Figure 6, 7). They were also observed in both the right and left postcerebrum. APGWamide-like immunoreactive fibres were present in the mesocerebrum neuropil and the connective nerves, as well as the penis nerve. Reconstruction of serial sections from a whole brain revealed that 47 right mesocerebral cells were labelled with neurobiotin, indicating their projections to the penis nerve. Twenty cells (43%) were found to contain APGWamide-like peptide (Table 2).

The cells in the right mesocerebrum retrogradely labelled from the NCPD also displayed APGWamide immunoreactivity (Figure 8). Double labelled cell bodies and fibres appeared in similar patterns to those backfilled from the penis nerve. Reconstruction of serial sections showed that 51 right mesocerebral cells extended to the NCPD. Nine cells (18%) contained APGWamide-like peptide (Table 2).

FMRFamide-like immunoreactive cell bodies and fibres

FMRFamide-like immunoreactive cell bodies were found in all ganglia in the central nervous system. Numerous FMRFamide immunoreactive fibres were also observed in every neuropil region as well as in the connective nerves.

Some of the retrogradely labelled cells from the penis

Figure 6. The left mesocerebrum immunoreacted with APGWamide antiserum. The image is a single optical section of a whole mount specimen. Note the APGWamide positive cells (yellow/green)(arrow).

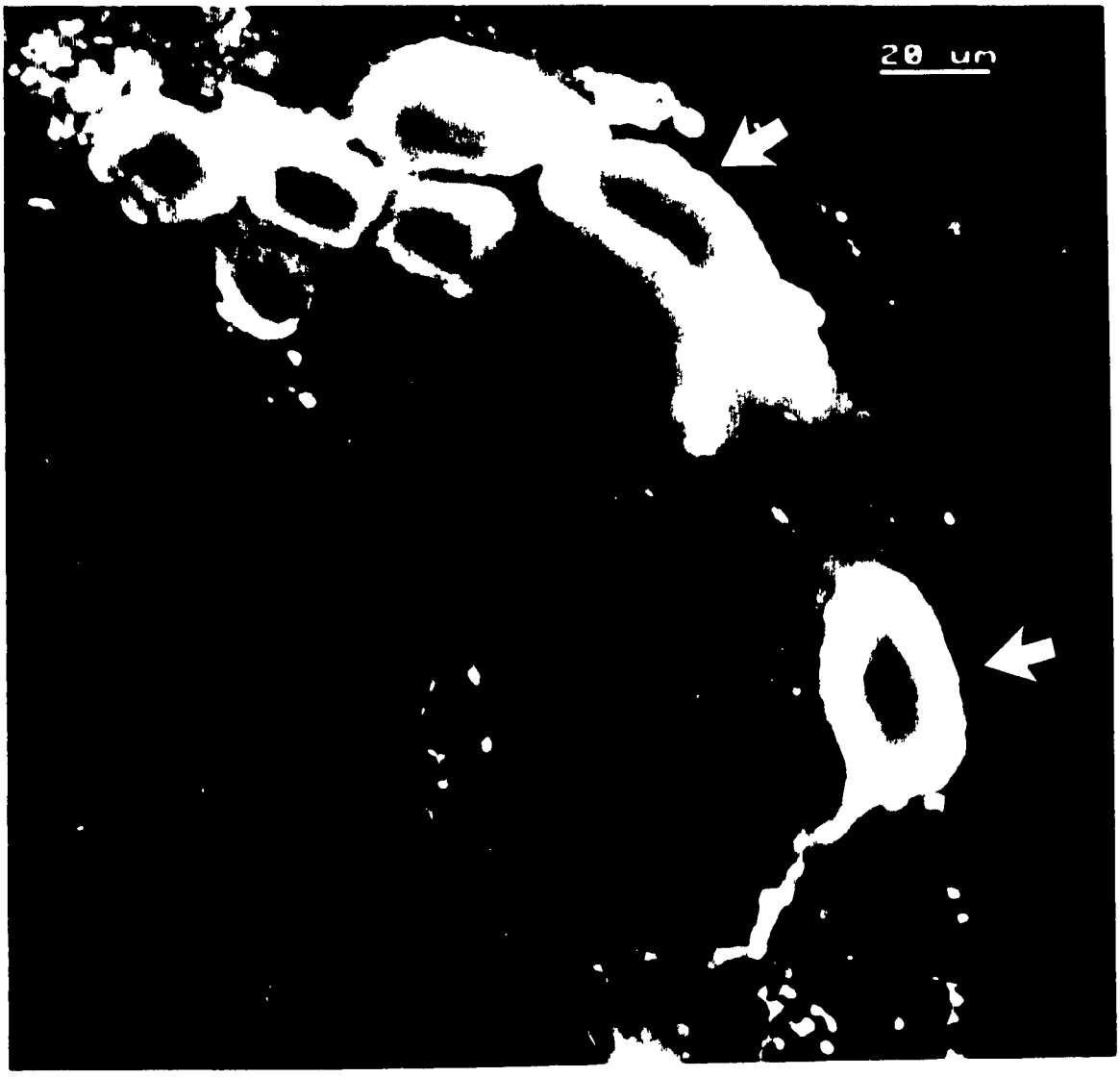
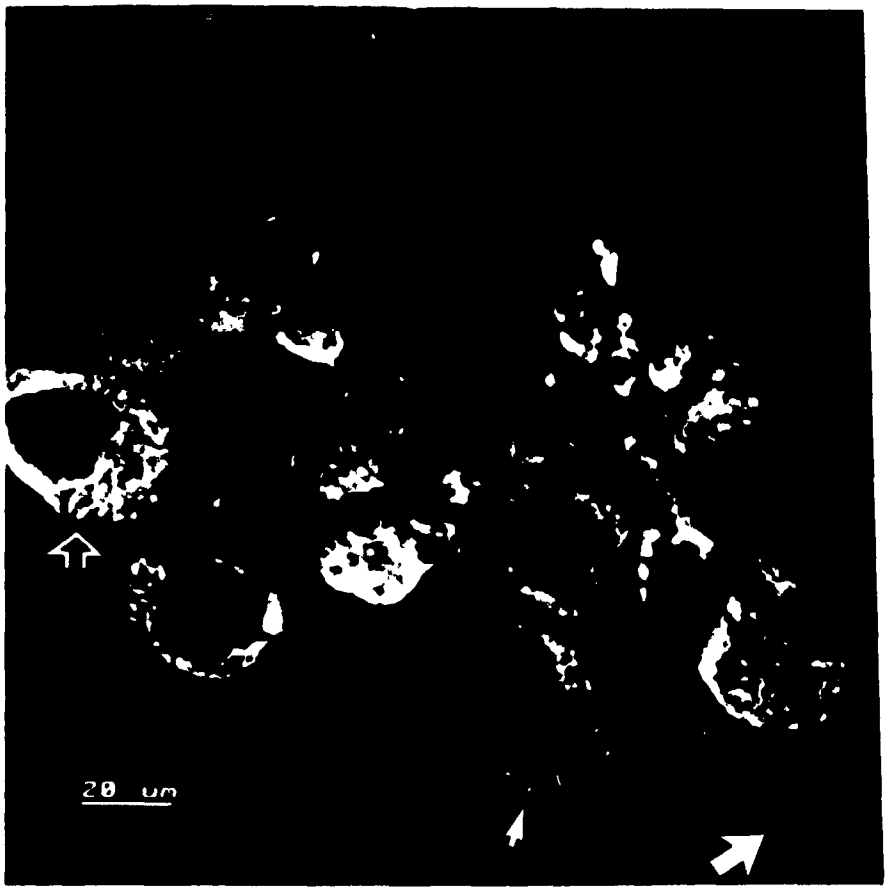

20 um

Figure 7. The right mesocerebrum backfilled from the penis nerve using Neurobiotin and immunoreacted with APGWamide antiserum.

Note the backfilled cells (red)(large arrow), and the APGWamide immunoreactive cells (yellow/green) (small arrow), as well as the double labelled cells (orange)(open arrow). The image is a composite of 13 optical sections scanned at intervals of 2 µm.

Figure 8. The right mesocerebrum backfilled from the NCPD using Neurobiotin and immunoreacted with APGWamide antiserum.

Note the backfilled cell (red)(large arrow), and the APGWamide positive cells (yellow/green)(small arrow), as well as the double labelled cells (orange)(open arrow). Composite of 13 optical sections scanned at intervals of 2 µm.


20 um

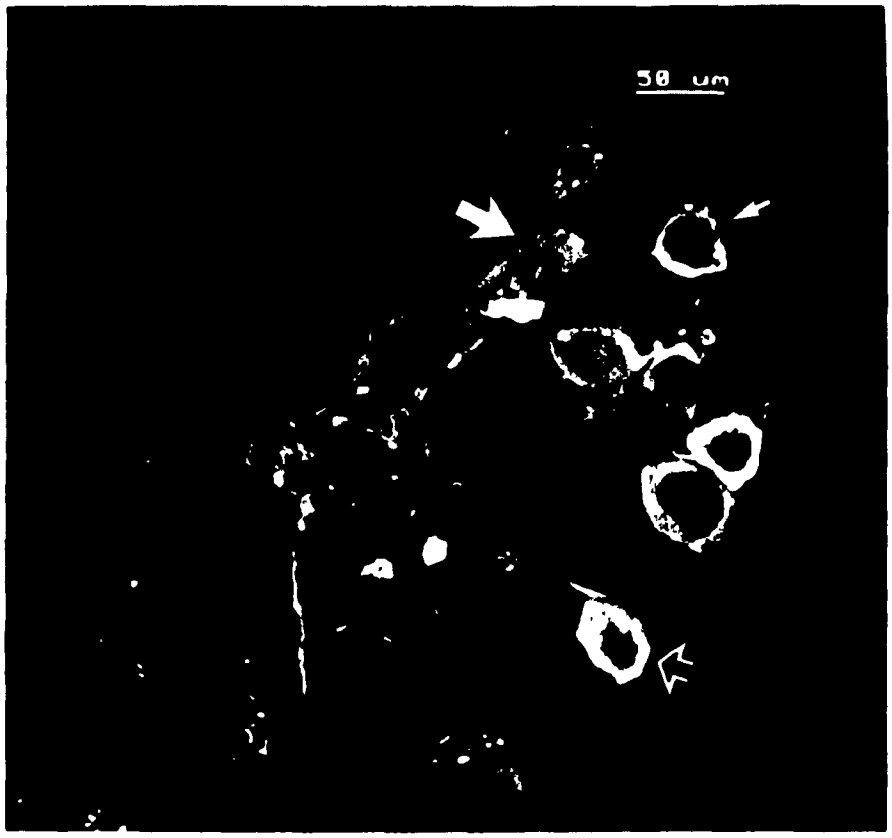

50 um

Table 2

APGWamide-like immunoreactive cell bodies in the right mesocerebrum also labelled by backfilling the penis nerve (PN) or the nervus cutaneus pedalis primus dexter (NCPD).

Backfilled Nerve	Number of Backfilled Cells	Number of APGWa Cells	Number of Backfilled Cells with APGWa	% Backfilled Cells With APGWa
PN	47	63	20	43%
NCPD	51	75	9	18%

nerve in the right mesocerebrum displayed FMRFamide-like immunoreactivity (Figure 9). Reconstruction from serial sections of a single brain revealed that 45 right mesocerebral cells backfilled from the penis nerve. Among these neurons, 12 cells (27%) were FMRFamide positive (Table 3).

Many retrogradely labelled cells from the NCPD in the right mesocerebrum also displayed FMRFamide-like immunoreactivity (Figure 10). Reconstruction of serial sections from a single brain revealed that 34 right mesocerebral cells projected to the NCPD. One-half of these cells (17 cells) contained FMRFamide-like peptide (Table 3). They accounted for 19% of the large FMRFamide immunoreactive cells (25-80 μm) in the right mesocerebrum.

From 8 pairs of adjacent sections immunoreacted with either APGWamide antiserum or FMRFamide antiserum, 70 mesocerebral neurons containing at least one peptide were visible in both sections. About half of these 70 cells contained both peptides. Examples are shown in Figure 11.

Preabsorption control

Preabsorption of the APGWamide antiserum with synthetic APGWamide completely abolished labelling (Figure 12). Likewise, preabsorption of the FMRFamide antiserum with synthetic FMRFamide also completely blocked labelling (Figure 13).

Peptide content of the anterogradely labelled cells

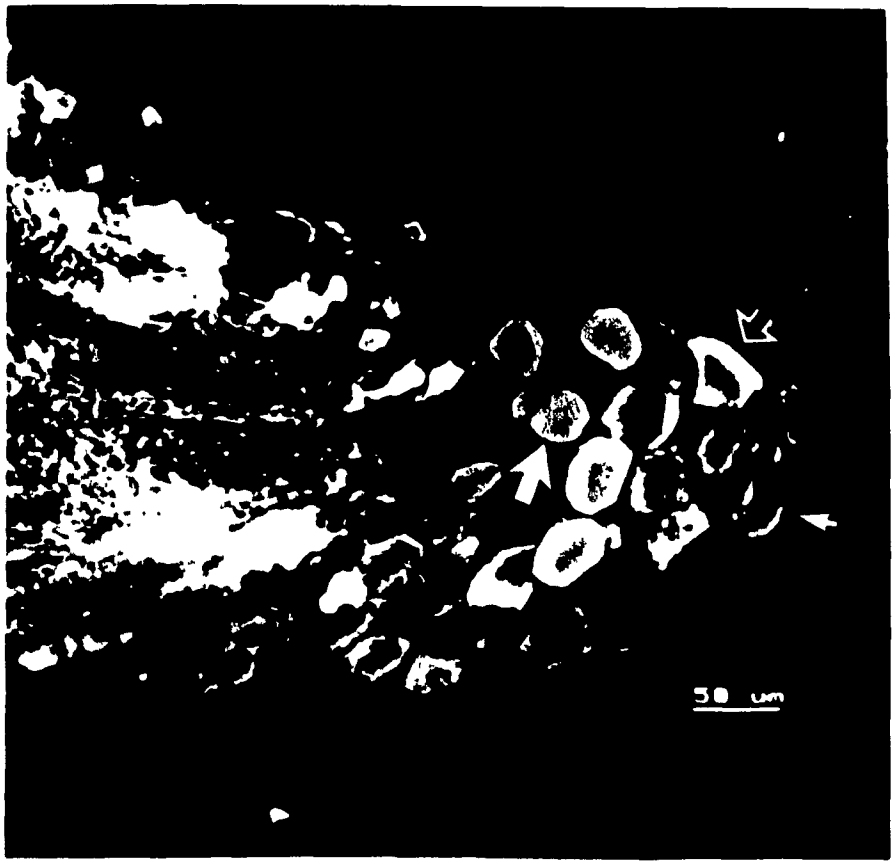
Among the 43 anterogradely labelled cells in the

Figure 9. The right mesocerebrum backfilled from the penis nerve using Neurobiotin and immunoreacted with FMRFamide antiserum.

Note the backfilled cells (red)(large arrow), and the FMRFamide positive cells (yellow/green)(small arrow), as well as the double labelled cells (open arrow). This image is a composite of 13 optical sections scanned at intervals of 2 µm.

Figure 10. The right mesocerebrum backfilled from the NCPD using Neurobiotin and immunoreacted with FMRFamide antiserum.

Note the backfilled cells (red)(large arrow) that is partially covered by another immunoreactive cell, and the FMRFamide immunoreactive cells (yellow/green) (small arrow), as well as the double labelled cells (orange)(open arrow). Composite of 10 optical sections at intervals of 2 µm.


50 um

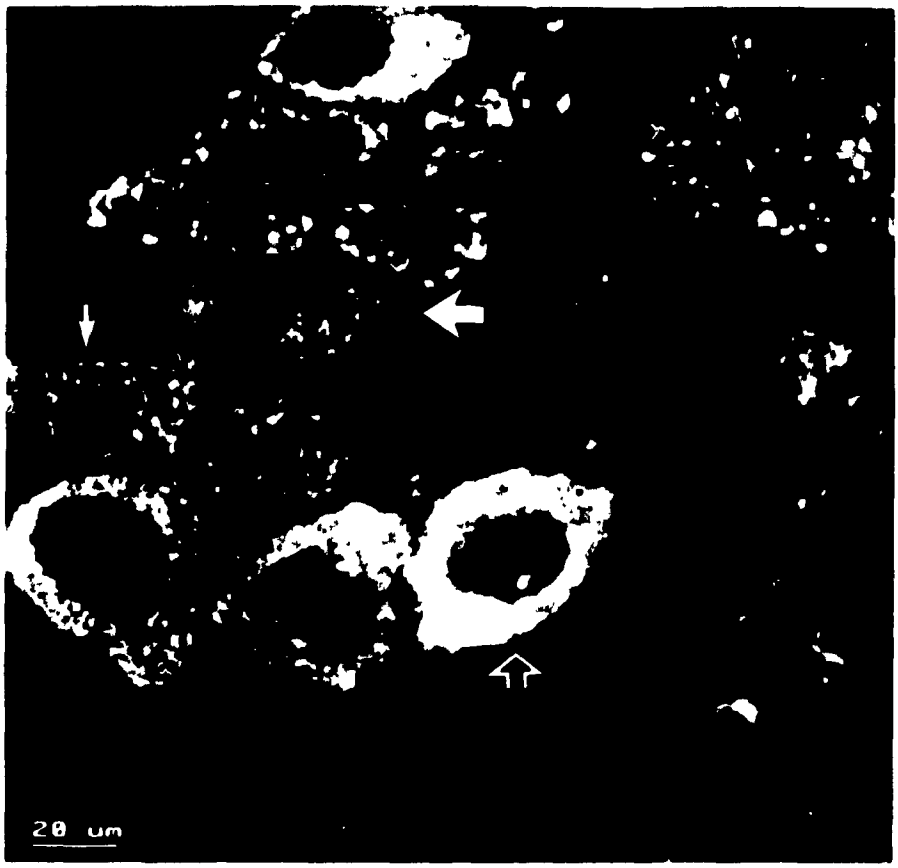

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Table 3

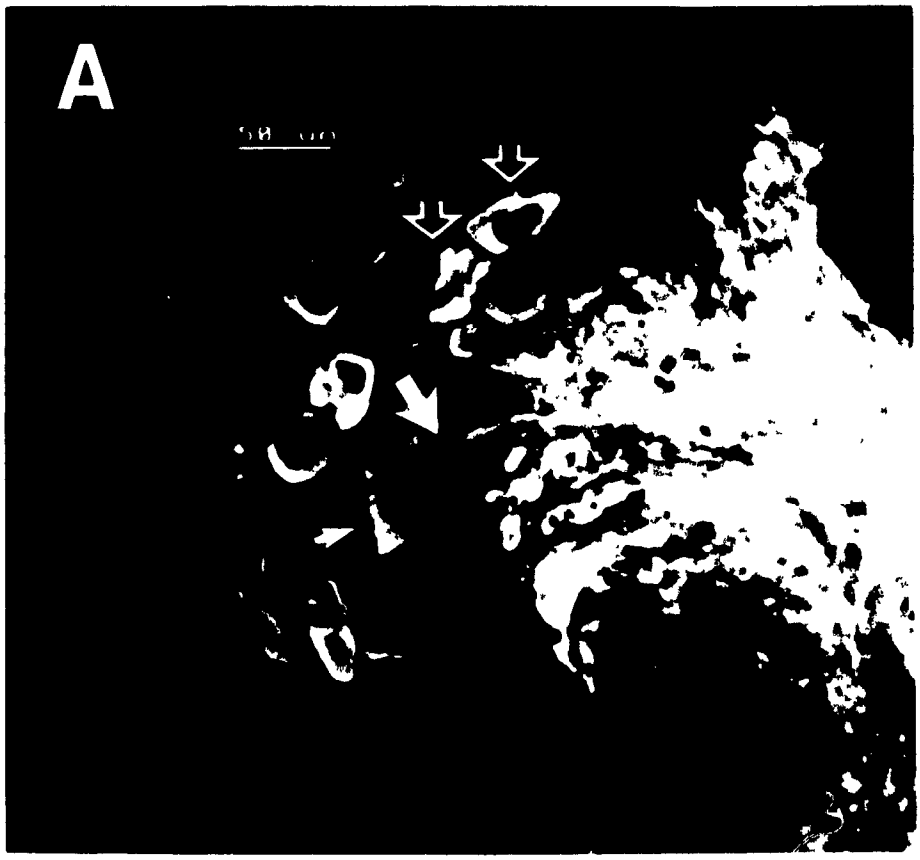
FMRFamide-like immunoreactive cell bodies in the right mesocerebrum also labelled by backfilling the penis nerve (PN) or the nervus cutaneus pedalis primus dexter (NCPD).

Backfilled Nerve	Number of Backfilled Cells	Number of FMRFa Cells	Number of Backfilled Cells with FMRFa	% Backfilled Cells With FMRFa
PN	45	62	12	27%
NCPD	34	91	17	50%

mesocerebrum, 16 cells were immunoreactive to APGWamide and/or FMRFamide antisera. Table 4 summarizes the axon projections of these cells and their peptide content. Eight out of sixteen neurons extend to the right CPC. Two of them contain both APGWamide-like and FMRFamide-like peptides. Among the three neurons projecting to the NCPD, two contain FMRFamide-like peptide. There are two neurons projecting to both the penis nerve and the NCPD. One neuron contains FMRFamide-like peptide only, while the other neuron contains both APGWamide-like and FMRFamide-like peptides. Figure 14 illustrates a mesocerebral neuron which projects to both the penis nerve and the NCPD. This neuron contains both APGWamide-like and FMRFamide-like peptides (Figure 15).

Figure 11. A pair of adjacent sections immunoreacted with either FMRFamide antiserum (A) or APGWamide antiserum (B).

Note the cell containing FMRFamide only (small arrows), and the cell containing APGWamide only (large arrows), as well as the cell containing both peptides (open arrows).


A

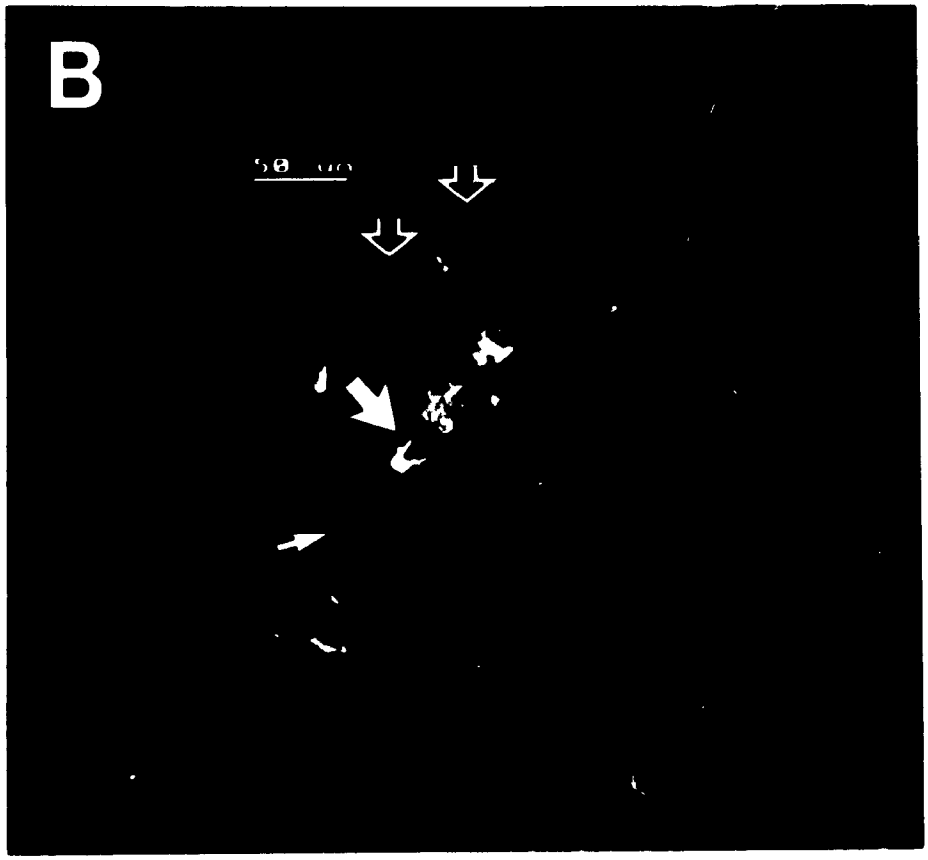

B

Figure 12. Preabsorption control for APGWamide immunoreactivity. (A) and (B) show adjacent serial sections (15 µm thick).

A. The right mesocerebrum immunoreacted with APGWamide antiserum. Note the APGWamide positive cells (green)(arrows).

B. Preabsorption of APGWamide antiserum with synthetic APGWamide completely abolished staining.

Scale bar = 50 µm.

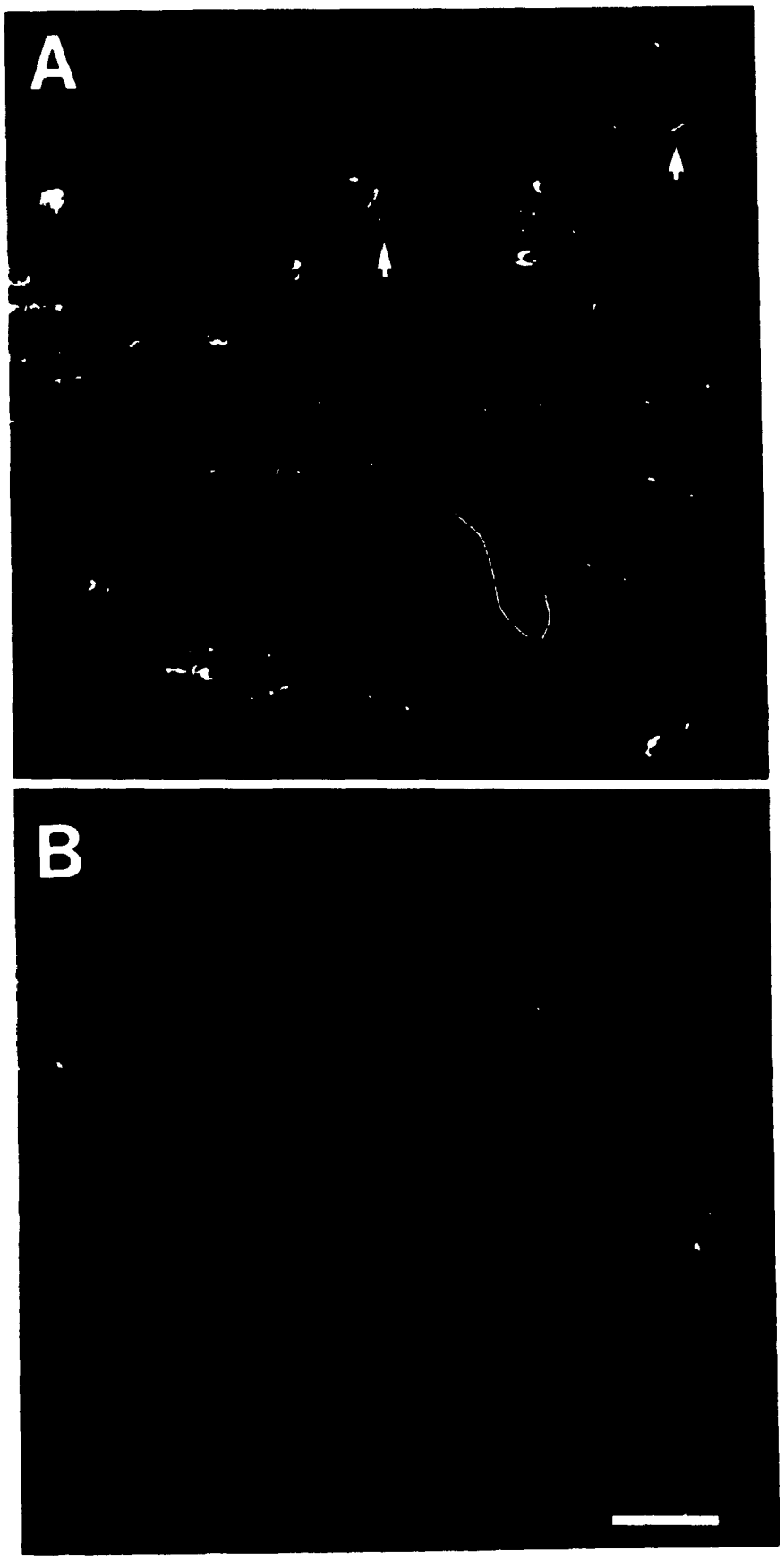

A
B

Figure 13. Preabsorption control for FMRFamide immunoreactivity. (A) and (B) show adjacent serial sections (15 µm thick).

A. The right mesocerebrum immunoreacted with FMRFamide antiserum. Note the FMRFamide positive cells (green)(arrows).

B. Section stained with preabsorbed FMRFamide antiserum. Note the absence of label.

Scale bar = 50 µm.

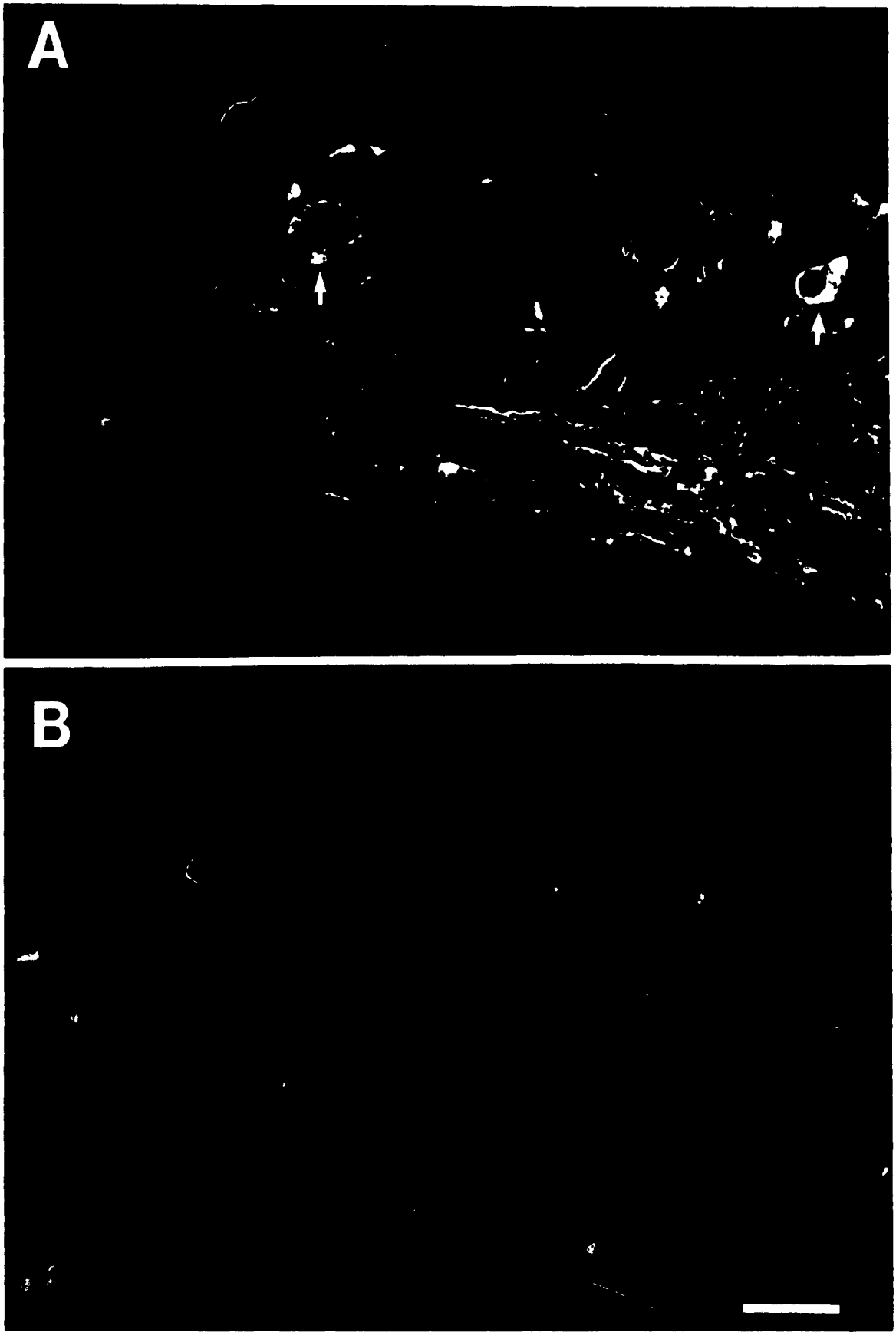

A
B

Table 4

Peptide contents and axon projections of injected neurons (n=16) in the right mesocerebrum.

Axon Projection[a]	APGWa alone	FMRFa alone	APGWa & FMRFa	No immuno-reactivity
CPC[b]	2	2	2	2
PN	2	1[c]	4[d]	0
NCPD	0	2[c]	1[d]	0

CPC, right cerebropedal connective.

NCPD, nervus cutaneus pedalis primus dexter.

PN, penis nerve.

[a] Some neurons have projections in the nerves additional to those listed.

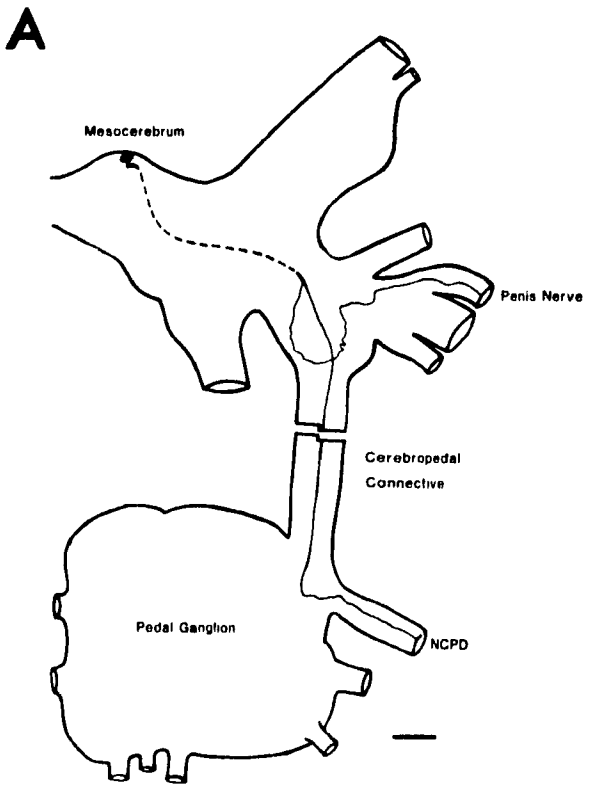
[b] Axon projecting to the CPC could not be traced to any further distance site.

[c] This number includes a neuron projecting to both the PN and the NCPD.

[d] This number includes a second neuron projecting to both the PN and the NCPD.

Figure 14. A. Drawing of a mesocerebral neuron injected with Neurobiotin. Scale bar = 200 µm.

B. Photograph of the right CPC and the NCPD. Note the axon running along the CPC and entering the NCPD (arrowheads). Scale bar = 100 µm.


A
Mesocerebrum
Penis Nerve
Cerebropedal
Connective
Pedal Ganglion
NCPD

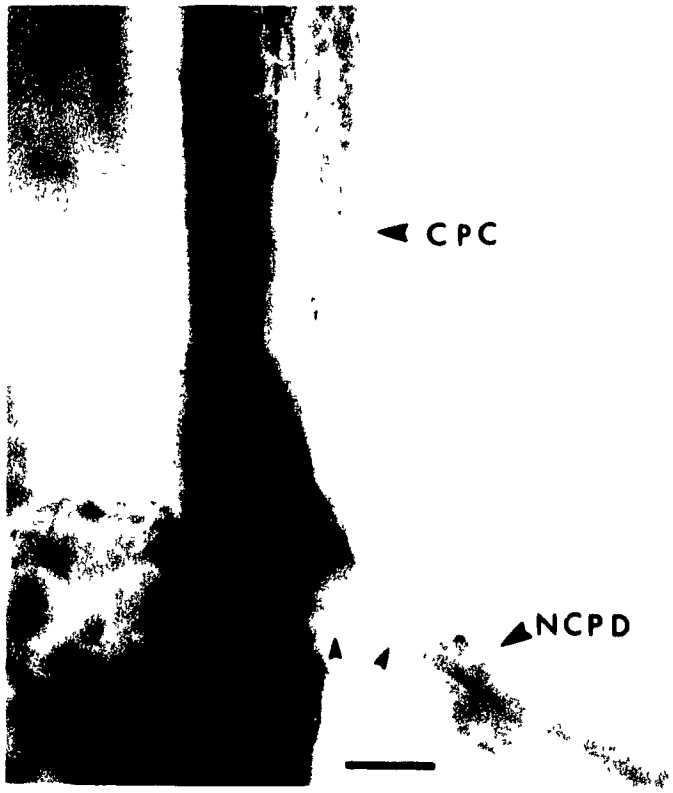
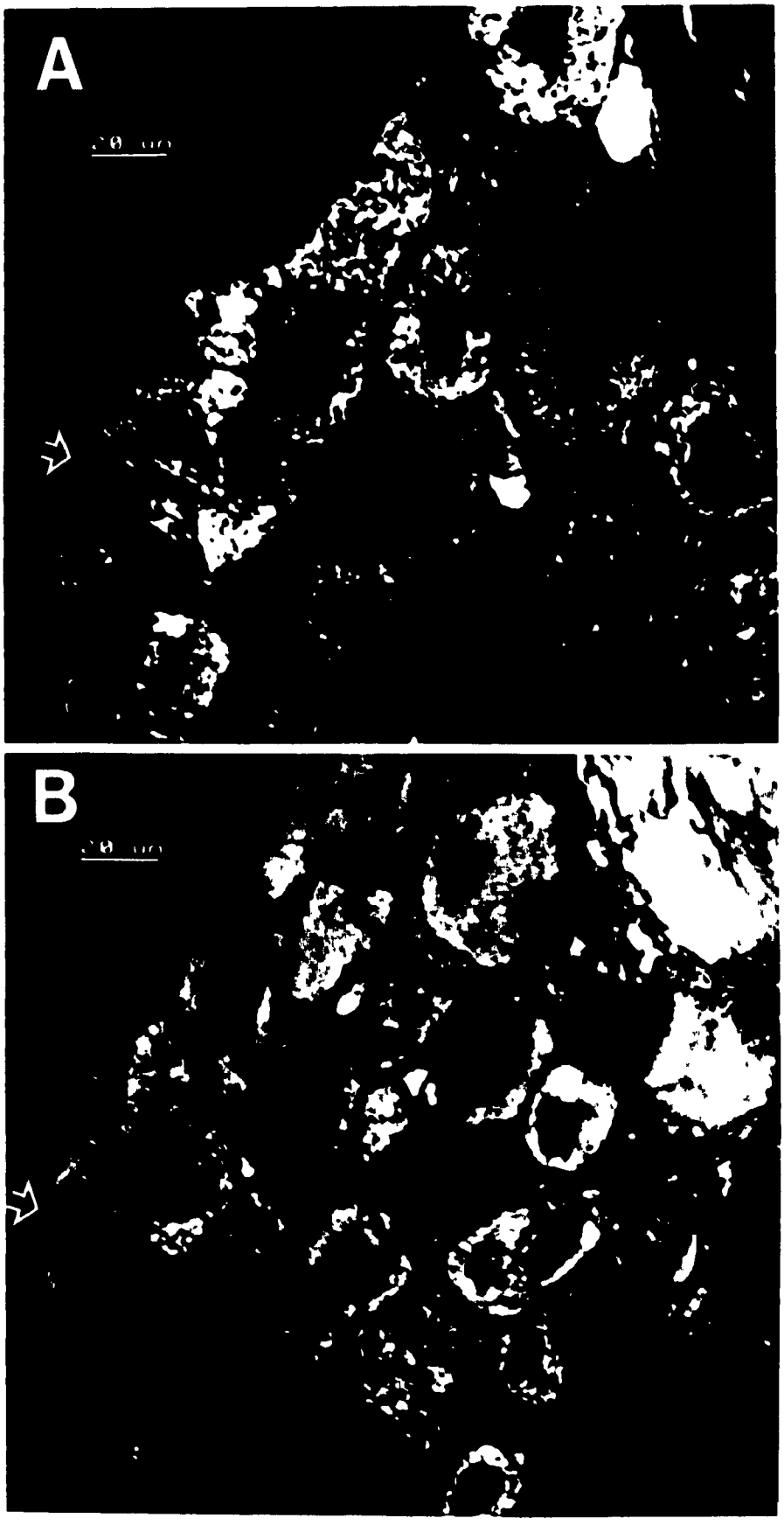

CPC
NCPD

Figure 15. The cell shown in Figure 14 contains both APGWamide-like and FMRFamide-like peptides.

A. The cell body of the injected cell immunoreacted with APGWamide antiserum. Note that the cell (open arrow) contains both Neurobiotin (red) and APGWamide (green). The extended focus image consists of 10 optical sections scanned at intervals of 2 µm.

B. The cell body of the injected cell stained with FMRFamide antiserum. Note that the cell (open arrow) contains both Neurobiotin (red) and FMRFamide (green). This image is a single optical section.


A
B

Chapter 4

Discussion

The results present in this study are significant in two aspects. First, it provides a better understanding of morphology of the mesocerebral neurons which play an important role in controlling penis eversion and dart shooting during courtship and mating. Second, it demonstrates that two peptides are present in the mesocerebral neuron. This preparation could serve as a model for studying interaction between peptides that co-localized in single neurons.

Anterograde labelling with Neurobiotin unequivocally revealed the axons of the right mesocerebral neurons up to 5 mm long. This labelling is certainly better than intracellular labelling with hexamminecobalt chloride, by which the axons of the right mesocerebral neurons were labelled to about 1.2 mm, i.e., just entering the origin of the right cerebropedal connective nerve (see Figure 2, Laberge and Chase, 1992). Anterograde labelling with Neurobiotin clearly provided more information on the morphology of the mesocerebral neurons. It not only confirmed earlier results that a majority of the mesocerebral cells have an axon in the right CPC (LaBerge and Chase, 1992, Chase, 1986), but also demonstrated, for the first time, that at least some of these axons enter the NCPD. It also indicated that the cells project to the NCSD and some cells have multiple projections (Figure 5). Another novel discovery is that many mesocerebral neurons have an axon branch in the penis nerve, which were not

detected in the earlier study (Chase, 1986). New phenomena that some mesocerebral cells project to the left medial lip nerve and the right peritentacular nerve were also observed (Table 1).

Whether the mesocerebral neurons that project to either the penis nerve or the NCPD are motorneurons remains unknown. Since stimulating the right mesocerebrum can cause the penis to contract (Chase, 1986), it is likely that the mesocerebral neurons directly innervate the penis muscle. This study shows that some mesocerebral neurons directly project to the NCPD. Therefore, they are perhaps the motorneurons innervating the dart sac. On the other hand, Some mesocerebral neurons project to the right CPC and terminate in the right pedal ganglion, where the motorneurons probably reside. The latter result provides additional evidence to support the assumption raised by Chase (1986), in which he reported that electrical stimulation of the NCPD was unable to produce antidromic response in the mesocerebral neurons tested and suggested that the motorneurons lie in the right pedal ganglion. Current results suggest that there are perhaps two clusters of motorneurons innervating the dart sac, i.e., the mesocerebral neurons and the right pedal neurons. As for the labelled cells in the right CPC and the right CPLC by backfilling the NCPD (Figure 4B, C), whether they are interneurons or motorneurons remains to be determined.

Contrary to the results of Griffond et al. (1992) in

which APGWamide positive neurons were found to locate in the right mesocerebrum but not in the left mesocerebrum, this study showed that APGWamide-like immunoreactive neurons were located in both anterior lobes of the right and the left mesocerebrum (Figure 6), as well as in the postcerebrum. However, more APGWamide-like immunoreactive neurons situated in the right anterior lobe than in the left anterior lobe. Since in this study, retrograde backfilling revealed that some of the left mesocerebral neurons had axons in the penis nerve, it is quite convincing that these left mesocerebral neurons also contain the APGWamide-like peptide as their counterparts in the right mesocerebrum. In comparison, there was evidence that APGWamide containing neurons were present in both the right and the left anterior lobes in *L. stagnalis*, even though in that case no left anterior lobe cells projected to the penis (Croll and Van Minnen, 1992). Croll and Van Minnen also reported that consistently strong hybridization signals of the probe for APGWamide encoding mRNA were observed in neurons of both the right and left lobes. Additionally, RNA blot analysis demonstrated that the expression of the APGWamide gene was more abundant in the right side than the left side of the central ganglia in *Lymnaea*, which is consistent with the asymmetrical distribution of penis neurons within the central nervous system (Smit et al., 1992). The mesocerebrum in *Helix* and the anterior lobe in *Lymnaea* are similar in many aspects. For example, in *Lymnaea*, the right anterior lobe is larger

than the left anterior lobe, which is partially due to the presence of a group of neurons, called the F cell, in the right anterior lobe. All F cells send processes into the penis nerve (Khennak and McCrohan, 1988).

Although the precise role of APGWamide-like peptide in *Helix aspersa* remains unknown, the APGWamide-like immunoreactive neurons in both the right and the left mesocerebrum are probably involved in copulation. APGWamide has been reported to inhibit spontaneous contractions of the crop and the tetanic contraction of the pharyngeal retractor muscle of the Japanese land snail *Euhadra congenita* (Kobayashi and Muneoka, 1990). In *L. stagnalis*, based on the facts that the cells in anterior lobe of the right cerebral ganglia project to the penis complex via the penis nerve and that APGWamide immunoreactive fibres are located in the penis retractor muscle (Croll, 1991), Croll et al. suggested that APGWamide may play a role in the relaxation of the penis retractor muscle during the eversion of the penis complex. In present study of *Helix aspersa*, the mesocerebral cells projecting to the penis nerve were found to contain APGWamide-like peptide. indicating that the APGWamide may play a similar role in this case.

Results in this study confirm the widespread distribution of FMRFamide immunoreactive neurons in the central nervous system, especially in the right mesocerebrum, as indicated by Elekes and Nässel (1990) and Marchand et al. (1991). Although no definite conclusions on

the function of FMRFamide in *Helix aspersa* can be drawn from the present results, it is clear that FMRFamide-like peptide may cause muscular contraction of the dart sac or involve in increasing heart rate and building up of the hydrostatic pressure, which in turn involves in expelling the dart sac during dart shooting. There is substantial evidence that FMRFamide can not only induce cardioexcition in *Helix* (Price, 1985), but also cause contraction in a number of smooth muscle, such as the tentacle muscle (Cottrell, 1983), and the anterior byssus retractor muscle in *Mytilus edulis* (Painter, 1982). It has been suggested that FMRFamide may act as a neurotransmitter or neurohormone in molluscs (Elekes and Nässel, 1990, Cottrell, 1989, Cooke and Gelperin, 1988). Cottrell (1989) suggested that at least some of the FMRFamide-like peptides exert their action via the circulation; one possible neurohaemoral release site is within the thick vascularized, connective tissue sheath which surrounds the central ganglia. On the basis of the discovery that in *Lymnaea*, axons of several FMRFamide positive neurons end on other neurons, muscle cell, and gland cells, Boer and co-workers (1984) suggested that FMRFamide is a neurotransmitter. In the present study, the extensive distribution of FMRFamide-like immunoreactive fibres in the neuropil regions of all the central ganglia and the peripheral nerves also indicates FMRFamide-like substances acting as central neurotransmitters or neurohormone or neuromodulator in *Helix aspersa*.

Current study clearly demonstrates for the first time that the right mesocerebral neurons projecting to either the penis nerve or the NCPD contain FMRFamide-like substance (Figure 9, 10). This result is consistent with the report by Cardot (1983). Cardot found that dart sac and penis, as well as flagellum, had thick muscular walls which displayed a rich FMRFamide immunoreactivity.

Similar to the right ventral lobe cells of *Lymnaea* that contain FMRFamide (Schot et al., 1984), most of the right postcerebrum cells in *Helix* are FMRFamide positive. Another similarity between *Helix* and *Lymnaea* is that both the right postcerebrum and the right ventral lobe have small cluster of APGWamide positive neurons (Croll et al., 1991). Therefore, one can assume that the right postcerebrum cells and the right ventral lobe cells are homogeneous and have similar function. Both the right postcerebrum cells and the right ventral lobe cells have axons in penis nerve, indicating they may involve in penis control.

FMRFamide antiserum used for the present study is likely to cross-react with the FMRFamide-like neuropeptides of *Helix aspersa* (See Price, 1986). It has been reported that seven FMRFamide-like peptides exist in *Helix* (Price et al., 1990, Cottrell, 1989). All of them are possibly stained with FMRFamide antiserum. In addition, the antiserum used in this study could possibly stain cells containing other peptides which are not members of the FMRFamide-like peptides but cross-react with FMRFamide

antiserum.

Cottrell et al. recently (1992) reported the surprising finding that the right mesocerebral cells cluster contained neither FMRFamide nor any FMRFamide-like peptides. They used radioimmune assays with antisera respectively selective for FMRFamide and hepta-FMRFamide-related peptides. The FMRFamide antiserum used in this study came from a different source than those used by Cottrell or other workers. It remains to be determined whether the true identity of substance labelled is FMRFamide. Although met-enkephalin immunoreactive cells were reported in both the right and the left mesocerebrum (Marchand et al., 1991) or only in the left mesocerebrum (Elekes et al., 1993), the peptide labelled by FMRFamide antiserum in the mesocerebrum should not be met-enkephalin since technical data provided by Peninsula Lab. indicate that 0% cross-reactivity exists between FMRFamide antiserum and met-enkephalin. In addition, the technical data would also seem to rule out neuropeptide Y or substance P as the real peptide labelled by FMRFamide antiserum since there is nearly nil cross-reactivity between these two substances and the antiserum.

Current results indicate that the mesocerebral neurons projecting to the penis contain APGWamide-like peptide, while the mesocerebral neurons projecting to the dart sac contain FMRFamide-like peptide, which is in general consistent with the hypothesis. Though the mesocerebral neurons backfilled from the penis nerve may contain

APGWamide-like peptide or FMRFamide-like peptide, and the neurons backfilled from the NCPD may contain FMRFamide-like peptide or APGWamide-like peptide. It appears that for the cells projecting to the penis nerve, a higher percentage of them contain APGWamide-like peptide (Table 2), while for the cells projecting to the NCPD, more of them contain FMRFamide-like peptide (Table 3). However, there is substantial overlap in the distribution of APGWamide-like peptide and FMRFamide-like peptide. In addition, some neurons contain both APGWamide-like peptide and FMRFamide-like peptide (Table 4). The peptide content of the mesocerebral neurons projecting to both the penis nerve and the NCPD or multiple directions was also observed in present study, which was not specified in the hypothesis. Although there are no quantitative data to confirm that the multiple projecting neurons are the neurons containing both APGWamide-like peptide and FMRFamide-like peptide, it is very likely to be the case.

Result shown in the Figure 15 demonstrates that in some cases, APGWamide-like peptide and FMRFamide-like peptide co-exist in a single mesocerebral neuron. As the APGWamide antiserum used in this study does not cross-react with FMRFamide (Van Minnen, J., personal communication), the two labelled substances unequivocally represent two (or two families of) different peptides. Considering some of the mesocerebral cells projecting to both the penis nerve and the NCPD, if APGWamide-like peptide is involved in penis

eversion and FMRFamide-like peptide is involved in dart shooting, it is not surprising that these multiple projection neurons have both peptides and each peptide is used in each circumstance. Strong evidence has proved that several modulatory peptides could colocalize in single neurons of *Aplysia* (Weiss et al., 1992).

Conclusions

Retrograde labelling shows that on the anterior surface of the mesocerebrum, 25-40 large neurons have axons projecting to the penis nerve and a similar number of neurons have axons projecting to the NCPD. Anterograde labelling reveals, for the first time, that the mesocerebral neurons project to the NCPD, the NCSD, the left medial lip nerve, the right peritentacular nerve, and the right pedal ganglion. Some mesocerebral neurons have multiple projections.

APGWamide immunoreactive substances exist in both the right and the left mesocerebrum. Quantitative analysis indicates that nearly half of the mesocerebral neurons projecting to the penis nerve contain APGWamide-like peptide and half of the mesocerebral neurons projecting to the NCPD contain FMRFamide-like peptide. On the other hand, FMRFamide-like peptide and APGWamide-like peptide were present in much smaller percentages of the cells projecting to the PN and the NCPD, respectively. Some mesocerebral cells contain both APGWamide-like peptide and FMRFamide-like

peptide.

Current results are consistent with the hypothesis that the mesocerebral neurons projecting to the penis contain APGWamide-like peptide, while the mesocerebral neurons projecting to the dart sac contain FMRFamide-like peptide. However, there is substantial overlap in the distributions of APGWamide-like peptide and FMRFamide-like peptide, and it remains unclear whether the mesocerebral neuron population can be characterized in terms of any consistent combination of peptide content and axon projection.

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